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DELIVERY OF TREFOIL PEPTIDES

The present invention relates to the field of *in vivo* protein delivery systems. More particularly, the present invention relates to the secretion *in vivo* of trefoil peptides by micro-organisms, preferably bacterial strains, preferably non-pathogenic strains, preferably non-invasive strains, preferably food grade strains, methods for delivering trefoil peptides using said systems and the use of said trefoil peptide expression systems for treatment of inflammatory disorders of the gastro-intestinal tract.

Lactococcus lactis is a Gram-positive non-pathogenic lactic acid bacterium which can survive in the intestine (Klijn *et al.*, 1995). It is not certain whether *L. lactis* can also be metabolically active in all of these environments.

The expression of tetanus toxin fragment C by *Lactococcus lactis* in view of vaccination was described by Wells *et al.* (1993b) and Robinson *et al.* (1997). Further, it was demonstrated that when preparations of *L. lactis* bacteria engineered to express either Interleukin-2 or Interleukin-6 together with tetanus toxin fragment C (TTFC) were administered intranasally to mice, more than 10 times more anti-TTFC was produced than after similar administration of strains expressing TTFC alone (International patent application published under WO 97/14806). These results prove the use of a cytokine-secreting, non-invasive experimental bacterial vaccine vector to enhance immune responses to a co-expressed antigen.

Trefoil peptides are secreted by epithelial mucus cells and are stable in an acid environment. These peptides contribute to the protection of the mucosa (formation of a gel over the epithelium) and are probably involved in the repair of damaged mucosa by stimulation of epithelial migration (Playford *et al.*, 1996). The production of trefoil peptides increases locally on places where damage occurs such as gastric ulcers and colitis (Wright *et al.*, 1990). Babyatsky *et al.* (1996) have shown that the administration of recombinant trefoil peptides reduces the damage at those places. In contradiction with most other proteins that are important for the protection of the mucosa (such as epidermal growth factor), most studies have demonstrated that trefoil peptides cause little or no proliferation, and

that they might even inhibit proliferation (Playford *et al.*, 1996). Three members of this family of trefoil peptides have been identified in humans and originally designated: pS2 (breast cancer oestrogen inducible gene), SP (spasmolytic peptide) and ITF (intestinal trefoil factor). In the present nomenclature pS2 is renamed as TFF1, SP as TFF2 and ITF as TFF3 (see e.g. Wong *et al.*, 1999). This new nomenclature will be used throughout the present text.

Both in humans, mice and rat TFF1 and TFF2 are predominantly found in the stomach while TFF3 is predominantly found in the duodenum and colon. Wong *et al.* (1999) give a recent overview of trefoil peptides. The contents of this article are incorporated by reference in the present disclosure.

The use of trefoil proteins or peptides for treating of disorders of and damage to the alimentary canal, including the mouth, oesophagus, stomach, and large and small intestine, as well as for the protection and treatment of tissues that lie outside the alimentary canal are described in WO 97/38712. These proteins can be used either to treat lesions in these areas or to inhibit the formation of lesions. These lesions can be caused by radiation therapy or chemotherapy for the treatment of cancer, any other drug including alcohol which damages the alimentary canal, accidental exposure to radiation or to a caustic substance, infection, a digestive disorder including but not limited to non-ulcer dyspepsia, gastritis, peptic or duodenal ulcer, gastric cancer, MALT lymphoma, Menetier's syndrome, gastro-oesophageal reflux disease, Crohn's disease, ulcerative colitis and acute colitis of chemical, bacterial or obscure origin.

Trefoil peptides are particularly useful to treat acute colitis.

ITF has also been used in combination with EGF (epidermal growth factor) for treating gastro-intestinal tract ulcers. *In vitro* and *in vivo* experiments have shown that the wound healing activities of EGF are markedly increased by treatment of EGF in combination with ITF, without increasing the proliferative action of EGF (Chinery and Playford, 1995).

Inflammatory bowel disease is the group name for a range of gastro-intestinal inflammations. Belonging to this group are enteritis, colitis, inflammations of respectively the mucosa of the duodenum or the colon. Crohn's disease (enteritis regionalis) and ulcerative colitis (colitis ulcerosa) are closely related, chronic and spontaneously recurring diseases of the gastro-intestinal tract. These

diseases are immunologically mediated and have environmental and genetic causes. Sartor (1995) describes the different aspects of inflammatory bowel disease. Crohn's disease has been particularly studied by for instance Herfath and Sartor, (1994), Cominelli *et al.* (1994), and MacDermott (1989).

The aim of the present invention is to provide a method for delivering trefoil peptides to treat gastro-intestinal disorders.

Another aim of the present invention is to provide a pharmaceutical composition for treating gastro-intestinal disorders.

The present invention relates more particularly to a micro-organism delivering a trefoil peptide *in vivo*. Preferentially said micro-organism is a bacterial strain, preferably a non-pathogenic strain, preferably a non-invasive strain, preferably a food grade strain, more preferably a gram-positive bacterial strain, most preferably a lactic acid fermenting bacterial strain, preferably a *Lactococcus* or a *Lactobacillus* species expressing a trefoil peptide *in vivo*.

It was not obvious from the capacity of *Lactococcus lactis* to deliver one heterologous antigen or its ability to produce molecules such as IL-2 and IL-6 *in vitro* and *in vivo* that bacteria would be an appropriate vehicle for delivery of other types of peptides or polypeptides *in vivo*. Further it is unknown whether said trefoil peptides expressed by said bacterial strains will provide a beneficial effect to inflammatory diseases of the gastro-intestinal tract, such as inflammatory bowel disease or acute colitis.

It is, therefore, surprising that it could be demonstrated in the present Examples section that bacterial strains are able to express trefoil peptides *in vivo* when present in the gastro-intestinal canal and exert a healing effect in acute colitis situations. By way of example, PCR fragments containing the coding region of rat TFF3 and mouse TFF1 were cloned. Recombinant vectors comprising these PCR clones under the control of a promotor and the *usp45 Lactococcus lactis* secretion signal sequence were constructed. Four transformed *Lactococcus lactis* strains were constructed which express either rat TFF3 or mouse TFF1 trefoil peptides. It was further shown in an *in vivo* mice model system that both recombinant rTFF3 and recombinant mTFF1 produced by these bacteria can surprisingly exert healing effects on the distal part of the inflamed colon.

According to a preferred embodiment, the present invention relates particularly to a bacterial strain delivering intestinal trefoil polypeptide (TFF3) *in vivo*.

According to another preferred embodiment, the present invention relates to a bacterium delivering TFF1 *in vivo*.

It is to be understood that the present invention also relates to micro-organisms parts or variants of any trefoil peptide. Said parts refer to biologically active parts which can be generated by methods known to those skilled in the art. These parts will generally contain at least 10 contiguous amino acids, typically at least 20 contiguous amino acids, more typically at least 30 contiguous amino acids, usually at least 40 contiguous amino acids, and preferably at least 50 contiguous amino acids. Said variants refer to variants which have the same biological activity as the above mentioned trefoil peptides.

It should also be clear that bacterial strains according to the present invention as defined above, may also express additional recombinant proteins which are beneficial to the treatment of any envisaged disorder.

According to another embodiment, the present invention relates to a method for the delivery of trefoil peptide to the gastro-intestinal tract comprising the administration of a micro-organism as defined above.

According to another aspect, the present invention also relates to the use of a micro-organism as defined above for the manufacture of an agent for the delivery of trefoil peptide to the gastro-intestinal tract.

According to yet another embodiment, the present invention relates to a pharmaceutical composition comprising a micro-organism expressing a trefoil peptide as defined above.

Advantageously, the pharmaceutical composition according to the present invention is preferably suitable for application to the mucosal surfaces.

Pharmaceutical compositions according to the present invention, and for use in accordance to the present invention, may comprise, in addition to the micro-organism, a pharmaceutically acceptable excipient, carrier, buffer, stabiliser or other materials well known to those skilled in the art. Such materials should be non-toxic and should not interfere with the efficacy of the active ingredient. The precise nature of the carrier or other material may depend on the route of

administration. Those of relevant skill in the art are well able to prepare suitable solutions.

According to another embodiment, the present invention relates to a method of treatment of gastro-intestinal inflammatory diseases comprising administration of a micro-organism delivering a trefoil peptide *in vivo* as defined above.

According to a preferred embodiment, the present invention relates to a method of treatment of gastro-intestinal inflammatory diseases comprising administration of a micro-organism delivering a TFF1 trefoil peptide *in vivo*.

According to another preferred embodiment, the present invention relates to a method of treatment of gastro-intestinal inflammatory diseases comprising administration of a micro-organism delivering a TFF3 trefoil peptide *in vivo*.

Administration of the micro-organism may be orally or by means of any other method known in the art allowing the micro-organism to enter the desired places to be treated, such as e.g. anal, vaginal. The micro-organism may be applied in a nutrient medium, i.e. a medium containing a substance or substances which sustain (at least *in vitro*) metabolic activity of the micro-organism. Such substances may sustain viability if not growth of the micro-organism. Such substances may include an energy source such as glucose, amino acids and so on.

The individual to which the micro-organism is administrated may be a human or an animal.

In a therapeutic context, i.e. where the biological effect of delivery of the polypeptide to an individual is beneficial to that individual, administration is preferably in a 'therapeutically effective amount', this being sufficient to show benefit to the patient. Such benefit may be at least amelioration of one symptom. The actual amount administered, and rate and time-course of administration, will depend on the aim of the administration, e.g. the biological effect sought in view of the nature and severity of the challenge and is the subject of routine optimisation. Prescriptions of treatment, for example decisions on dosage etc, is within the responsibility of general practitioners and other medical doctors.

A composition comprising micro-organisms according to the present invention may be administered in accordance with the present invention alone or in combination with other treatments, either simultaneously or sequentially.

According to another embodiment, the present invention relates to the use of a micro-organism expressing a trefoil peptide as defined above for the preparation of a medicament for the treatment of acute phases of diseases of the gastro-intestinal tract or diseases involving lesions at mucosal surfaces.

These diseases are preferably acute gastro-intestinal disorders of chemical, bacterial or obscure origin. Belonging to this group are enteritis, colitis, inflammations of, respectively, the mucosa of the duodenum or the colon. Also included is traveller's disease. Crohn's disease (enteritis regionalis) and ulcerative colitis (colitis ulcerosa) are closely related, chronic and spontaneously recurring diseases of the gastro-intestinal tract.

The disease states to be treated can also include disorders of and damage to the alimentary canal, including the mouth, oesophagus, stomach, and large and small intestine, as well as for the protection and treatment of tissues that lie outside the alimentary canal. The trefoil proteins expressed by the bacterial strains according to the present invention can be used either to treat lesions in these areas or to inhibit the formation of lesions. These lesions can be caused by: radiation therapy or chemotherapy for the treatment of cancer, any other drug including alcohol which damages the alimentary canal, accidental exposure to radiation or to a caustic substance, infection, a digestive disorder including but not limited to non-ulcer dyspepsia, gastritis, peptic or duodenal ulcer, gastric cancer, MALT lymphoma, Menetier's syndrome, gastro-oesophageal reflux disease, and Crohn's disease.

According to a preferred embodiment, the present invention relates to a bacterial strain delivering a trefoil peptide as defined above for use in the preparation of a medicament for treating acute colitis, including but not limited to acute flare-ups in Crohn's disease and ulcerative colitis.

According to another embodiment, the present invention relates to a method for producing a micro-organism delivering a trefoil peptide *in vivo* as defined above comprising transforming a micro-organism with a recombinant

vector carrying a trefoil polypeptide coding sequence under the control of a suitable promoter and a suitable bacterial secretion signal sequence.

Said bacterial secretion signal sequence can be any sequence known in the art to perform said function. Preferably, for *L. lactis* said secretion signal is the *usp45 L. lactis* secretion signal sequence. Said promoter sequence can be any promoter allowing expression of said coding sequence in said micro-organism. Examples given in the examples section include the known inducible *E. coli* phage T7 promoter and the known constitutive P1 promoter of *L. lactis*.

The present invention also relates to a recombinant vector comprising at least part of trefoil peptide coding sequence under the control of a suitable promoter and a suitable secretion signal sequence. Said recombinant vector can be used to deliver *in vivo* at least part of a trefoil peptide sequence which can exert on healing effect on damaged areas of the mucosal surfaces.

The following examples merely serve to illustrate the present invention, and are not to be construed as limiting the invention in any way.

All documents mentioned in this text are incorporated by reference.

FIGURE LEGENDS

Figure 1: Overview of the plasmids used.

Figure 1a : Schematic maps of the plasmids pL2rTFF3, pL2mTFF1v1, pT1rTFF3 and pT1mTFF1. T7 is the major late promoter from coliphage T7 (Studier and Moffatt, 1986). P1 is the lactococcal promoter as in Waterfield *et al.*, (1995), usp45S is a DNA fragment encoding the secretion signal peptide from the lactococal Usp45 protein (van Asseldonck *et al.*, 1990), rtff3 is a DNA fragment encoding the mature part of rat TFF3, mtff1 is a DNA fragment encoding the mature part of murine TFF1, mtff1v1 is a DNA fragment encoding a truncated (missing two aminoterminal aa residues) mature murine TFF1, Cm is the chloramphenicol selection marker, Em is the erythromycin selection marker.

Figure 1b: DNA sequence of plasmid pL2rTFF3 (SEQ ID NO 1).

Figure 1c: DNA sequence of plasmid pL2mTFF1v1 (SEQ ID NO 2).

Figure 1d: DNA sequence of plasmid pT1rTFF3 (SEQ ID NO 3).

Figure 1e: DNA sequence of plasmid pT1mTFF1 (SEQ ID NO 4).

Figure 2: SDS-polyacrylamide gel electrophoresis. The different protein fractions are derived from the medium of *L. lactis* MG1820 [pILPOL] (control), MG1820 [pILPOL; pL2mTFF1v1] or MG1820 [pILPOL; pL2rTFF3] cells. The left lane contains marker proteins wherein the molecular weight is given in kDa. The proteins were visualised using Coomassie Blue staining.

Figure 3: Representation of the histological scores of the middle part of the colon. Top left hand side graphic: epithelium damage (middle part colon). Top right hand side graphic: inflammatory infiltration (middle part colon). Bottom graphic: sum of the histological scores of the top graphics (middle part colon).

Figure 4: Representation of the histological scores of the distal part of the colon. Top left hand side graphic: epithelium damage (distal part colon). Top right hand side graphic: inflammatory infiltration (distal part colon). Bottom graphic: sum of the histological scores of the top graphics (distal part colon).

EXAMPLES

Description of the general methods used in this invention

Culture media

GM17 is M17 (Difco, Detroit) supplemented with 0.5 w/v % of glucose. M9 medium contains per litre: 6g of Na_2HPO_4, 3 g of KH_2PO_4, 1 g of NH_4Cl, 0.5 g of NaCl, 2 mmol of $MgSO_4$, 0.1 mmol of $CaCl_2$ and 5 g of Casitone (Difco). M9B is M9 supplemented with 2.1 g of $NaHCO_3$ and 2.65 g of Na_2CO_3 per liter. GM9B is M9B supplemented with 0.5 w/v % of glucose. LM9B is M9B supplemented with 0.5 w/v % of lactose.

When appropriate the antibiotics, erythromycin (Er) or chloramphenicol (Cm), were added to the respective media at final concentrations of 5 µg/ml each. The designation used to indicate the presence of antibiotic is, e.g. GM17Er, LM9BCm and so on. Solid media contained 1.2 % agar.

Recombinant DNA techniques

DNA modifying enzymes and restriction endonucleases were used under standard conditions and in the buffers recommended by the manufacturers. General molecular cloning techniques and the electrophoresis of DNA and proteins were carried out according to standard procedures. *L. lactis* was transformed by electroporation of cells grown in the presence of glycin (Wells *et al.*, 1993a). Plasmid DNA was routinely purified using the Qiagen Plasmid Kit

PCR amplification of mouse TFF1 (mTFF1)

The PCR reaction was carried out on plasmid pGEMmpS2 (kindly provided by Dr. Marie-Christine RIO, Institut de Génétique et Biologie Moléculaire et Cellulaire, Illkirch Cedex, France) using the oligonucleotide primers mTFF1S and mTFF1A. The pGEMmpS2 plasmid was first digested with *Hin*dIII to obtain a linear fragment. The mTFF1S primer corresponds to the first 18 nucleotides of the sense strand of *mTFF1* from the first nucleotide behind the signal sequence. The mTFF1A primer is complementary to the last 26 nucleotides of the sense strand of *mTFF1* including the stop codon, and introduces an extra *Spe*I restriction site.

mTFF1S: 5'-CAGGCCCAGCCCAGGCC –3' ((SEQ ID NO 5)

mTFF1A: 5'-GCACTAGTTAGAAGGGACATTCTTCTTCTTG AG-3' (SEQ ID NO 6) wherein ACTAGT in mTFF1A represents an *Spe*I site

PCR amplification was carried out using Vent™ DNA polymerase (New England Biolabs (Beverly, USA) which gives a PCR product carrying blunt ends. The PCR mixture consisted of 2 units Vent DNA polymerase, 10µl Vent buffer (thermopol), 4µl dXTP's (0.5mM maximum), 5µl (0.5µM) of each primer, 1µl (50 ng) template DNA and 74µl H_2O. Six reactions were set up differing in their final concentration of $MgSO_4$, adjusted to 0, 1, 2, 3, 4 and 5 mM respectively. PCR amplification cycles were: T_0 for 300" at 94°C, T_1 for 45" at 94°C, T_2 for 30" at 60°C, T_3 for 20" at 72°C, T_4 for 10" at 20°C. These cycles T_1 until T_3 were carried out 30 times.

PCR amplification with these primers rendered the gene for mature *mTFF1* lacking the signal sequence and including an additional *Spe*I restriction site. After checking by gel electrophoresis, the amplified fragment appeared as a band in the expected length range. The 5' end of the mTFF1 sequence contains two possible target sequences complementary to the forward primer. As a consequence two fragments of 202 base pairs and 208 base pairs respectively can be amplified from the mTFF1 cDNA by use of the mentioned primers. These fragments are not expected to be resolved by agarose gel electrophoresis.

PCR amplification of rat TFF3 (rTFF3)

The PCR reaction was carried out on plasmid pBluerITF (kindly provided by Dr. Daniel K. Podolsky, Massachusetts General Hospital, Boston, USA) using the oligonucleotide primers rTFF3S and rTFF3A. The rTFF3S primer corresponds to the first 23 nucleotides of the sense strand of *rTFF3* from the first nucleotide behind the signal sequence. The rTFF3A primer is complementary to the last 23 nucleotides of the sense strand of *rTFF3* including the stop codon, and introduces an extra *Spe*I restriction site.

rTFF3S: 5'-CAGGAATTTGTTGGCCTATCTCC-3' (SEQ ID NO 7)

rTFF3A: 5'-CGACTAGTTAAAATGTACATTCTGTCTCTTGCA-3' (SEQ ID NO 8) wherein ACTAGT in rTFF3A represents an *Spe*I site

The trinucleotide TTA in the antisense primer introduces a TAA stop codon which is different from the wild-type *rTFF3* stop codon (TGA). PCR amplification conditions were as described before under "PCR amplification of mouse TFF1 (mTFF1)".

PCR amplification with these primers rendered the gene for mature *rTFF3* lacking the signal sequence, with an altered stop codon and including an additional *Spe*I restriction site. Following agarose gel electrophoresis and EtBr staining a band having the expected size range (187 bp) was detected.

Construction of plasmids

Two different types of vectors were used as acceptors for either the mTFF1 or rTFF3 trefoil peptide encoding PCR fragments. The primary structure of the two parental vectors - pT1NX, derived from pTREX1 (Wells and Schofield, 1996), and pLET2NX, derived from pLET2N (Steidler *et al.*, 1995) - contains the following common elements: a promoter (T7 or P1), the *L.lactis usp45* secretion signal sequence (van Asseldonk *et al.*, 1990 and European patent application published under No. 0 455 280), modified to contain a *Nae*I restriction site overlapping the sequence encoding the ultimate aa residue (Steidler *et al.*, 1995), and a

downstream *Spe*I restriction site. pT1NX derived plasmids specify resistance to erythromycin; pLET2NX derived plasmids specify resistance to chloramphenicol. The PCR fragments were treated for 1 hour at 37°C using 50µl DNA solution, 10µl *Spe*I-buffer, 50 units *Spe*I, 10 units T4 polynucleotide kinase (Gibco BRL, Bethesda, USA), 0.5 mM ATP, adjusted to pH 7.5, and 36µl H_2O. The vector pT1NX was digested for 1 hour at 37°C using 10 à 20µl purified DNA, 10µl *Nae*I buffer, 10 units *Nae*I, 50 units *Spe*I, 1 unit calf intestine alkaline phosphatase (Boehringer, Mannheim, Germany) and 73 à 63µl H_2O. After 30 minutes incubation, 50 units of *Spe*I and 10 units of *Nae*I were again added to the mixture. The restriction enzymes were inactivated and extracted from the mixture by phenol/chloroform extraction. After restriction digestion, the mTFF1-derived band (comprising a 195 bp and a 201 bp fragment as described before under "PCR amplification of mouse TFF1 (mTFF1)", the 180 bp rTFF3 - derived fragment and the vector parts were excised from the agarose gel. Following ligation of the respective PCR fragments and the vector for 45 minutes at 16°C using "Ready To Go" T4 DNA ligase (Pharmacia Biotech, UK) recombinant plasmids were obtained containing the mTFF1 and the rTFF3 cistrons as an in-frame fusion to the *usp45* secretion signal sequence under the control of the promoter.

The plasmids pT1mTFF1 and pT1rTFF3 (Figure 1a), which contain the constitutive *L. lactis* P1 promoter, resulted from ligation of the purified *Nae*I - *Spe*I vector part of pT1NX and the *Spe*I cut and 5' phosphorylated PCR fragments.

The plasmids pL2mTFF1v1 and pL2rTFF3 (Figure1a), which contain the inducible *E. coli* phage T7 promoter, resulted from ligation of the purified *Nae*I - *Spe*I vector part of pLET2N and the *Spe*I cut and 5' phosphorylated PCR fragments. The T7 promoter can only be activated by the cognate T7 RNA polymerase encoded by e.g. plasmid pILPOL. This plasmid is present in *L. lactis* strain MG1820 [pILPOL] (Wells *et al.*, 1993c).

For structural analysis plasmid pT1mTFF1 and pT1rTFF3 were transformed into *L. lactis* strain MG1363. The cells were grown on GM17Er plates. Colonies were grown in 2.5 ml GM17Er and the plasmids were isolated. By means of an analytical digest, the restriction pattern of the pT1NX vector (2µl DNA (pT1NX), 20 units *Eco*RI, 50 units *Spe*I, 2µl *Spe*I-buffer and 15µl H_2O) and the isolated recombinant plasmids (5µl DNA, 20 units *Eco*RI, 50 units *Spe*I, 2µl *Spe*I-buffer,

0.25 µl of a 10 µg/ml Rnase A stock solution, 12µl H_2O) were compared. The plasmids were cut with *Eco*RI and *Spe*I for 1h at 37°C. In the reference plasmids, two linear fragments of 907bp and 4999bp are predicted. In pT1mTFF1, two bands of 499 bp and 4999 bp are predicted. In pT1rTFF3, two bands of 478 bp and 4999 bp are predicted. The sizes of the experimentally obtained fragments, as visualized by agarose gel electrophoresis and EtBr staining, were consistent with the predicted lengths. From each recombinant plasmid, one positive culture was striked out on GM17Er plates to obtain isolated colonies. One colony was subsequently inoculated in 100 ml GM17Er medium and grown to saturation. The cells were collected and the plasmids were purified. Their physical structure was verified by restriction enzyme analysis and agarose gel electrophoresis. In addition, sequence analysis revealed that the *mTFF1* and *rTFF3* cistrons had been ligated perfectly in frame with the *usp45* secretion leader sequence. pT1mTFF1 contains a 208 bp insert which represents the complete coding sequence of mature mTFF1 (as described before under "PCR amplification of mouse TFF1 (mTFF1)"). pT1rTFF3 contains a 180 bp insert encompassing the complete coding sequence of mature rTFF3.

For structural analysis plasmids pL2mTFF1v1 and pL2rTFF3 were transformed into strain MG1820[pILPOL]. The cells were grown on GM17Cm plates. Colonies were grown in 2.5 ml GM17Cm and the plasmids were isolated. By means of an analytical digest, the restriction pattern of the pLET2NX vector (2µl DNA (pLET2NX), 20 units *Eco*RI, 50 units *Spe*I, 2µl *Spe*I-buffer and 15µl H_2O) and the isolated recombinant plasmids (5µl DNA, 20 units *Eco*RI, 50 units *Spe*I, 2µl *Spe*I-buffer, 0.25 µl of a 10 µg/ml Rnase A stock solution, 12µl H_2O) were compared. The recombinant plasmids were cut with *Eco*RI and *Spe*I for 1h at 37°C. In the reference plasmids, two linear fragments of 907bp and 4650bp are predicted. In pL2mTFF1, two bands of 499 bp and 4650 bp are predicted. In pL2rTFF3, two bands of 478 bp and 4999 bp are predicted. The sizes of the experimentally obtained fragments, as visualized by agarose gel electrophoresis and EtBr staining, were consistent with the predicted lengths. From each recombinant plasmid, one positive culture was striked out on GM17Cm plates to obtain isolated colonies. One colony was subsequently inoculated in 100 ml GM17Cm medium and grown to saturation. The cells were collected and the

plasmids were purified. Their physical structure was verified by restriction enzyme analysis and agarose gel electrophoresis. In addition, sequence analysis revealed that the *mTFF1* and *rTFF3* cistrons had been ligated in frame with the *usp45* secretion leader sequence. The analysis further showed that pL2mTFF1v1 contains a 202 bp insert (consequently missing the first two aminoterminal aa residues of mature mTFF1 ; as described before under "PCR amplification of mouse TFF1 (mTFF1)". pL2rTFF3 contains a 180 bp insert encompassing the complete coding sequence of mature rTFF3.

The sequences of the recombinant plasmids are given in figures 1b to 1e. Their complete sequences were compiled from the published sequences of the constituting parts. In addition, relevant sections of the sequences such as PCR fragments and ligation junction points were experimentally verified.

Protein expression in transformed L. lactis

L. lactis strains were transformed with the plasmids as constructed above. For transformation of the pT1mTFF1 and the pT1rTFF3 plasmids, *L. lactis* strain MG1363 (Gasson, 1983) was used. For transformation of the pL2mTFF1v1 and the pL2rTFF3 plasmids, L. lactis strain MG1820 (pILPOL) (Maeda and Gasson, 1986) was used.

The expression of the proteins by these transformed *L. lactis* strains was detected by SDS-PAGE.

To prepare culture supernatant fractions, the cells were grown for 20 hours at 28°C in five ml GM17Er medium for pT1-type plasmids or GM17Cm medium for pL2-type plasmids. The cultures were diluted 1/100 in five ml of either GM17Er or GM17Cm medium and grown for 3 hours at 28°C. The cells were collected by centrifugation at 2800 rpm for 20 min and resuspended in five ml of the appropriate medium, i.e., GM9BEr for MG1363 cells or LM9BCm for MG1820 [pILPOL] cells. After a further five hours of growth the cells were pelleted. The proteins present in the medium fractions were recovered by phenol extraction and ethanol precipitation.

The proteins expressed in the culture supernatant fraction of a *L. lactis* MG1820 control strain compared to *L. lactis* MG1820 strains transformed with

[pILPOL; pL2mTFF1v1] or [pILPOL; pL2rTFF3] are shown in Figure 2. As can be observed from this figure, the expression of the recombinant genes is quite low. This renders the observed *in vivo* result surprising since others use purified trefoil peptides in therapies for the repair of gastric and intestinal injury at dramatically higher levels; e.g. Tran *et al.* (1999) used daily intrarectal application of human recombinant TTF2 at levels of 2.5 mg/kg body weight for five days to obtain a reduction in the inflammatory index of experimentally installed colitis in rats (intracolonic administration of dinitrobenzene sulphonic acid in alcohol).

Preparation of cells for oral administration

Transformants of *L. lactis* strains, MG1363 [pTREX1], MG1363 [pT1mTFF1] and MG1363 [pT1rTFF3] were streaked on GM17Er plates and grown overnight at 28°C. In each case a single colony was subsequently grown overnight at 28°C in 15 ml GM17Er medium. To this culture, 15 ml 100% glycerol was added in order to preserve said cells at –20°C. Each day, the necessary amount of cells could be inoculated for treatment of mice. To this end the culture was diluted 1/200 into 10 ml GM17Er medium. After minimum 20 hours of growth at 30°C, the cells were collected by centrifugation for 15 min at 2800 rpm. The cells were then resuspended in 1 ml M9B without antibiotic.

From this culture 100 µl (containing a minimum of 1.10^8 cells) were administrated intragastric per mouse.

In vivo tests in mice with acute colitis

The effect of the trefoil peptides expressed from these *L. lactis* bacteria was tested out in mice suffering from acute colitis. Twenty-one female Balb/c mice received 5% DSS (dextrane sodium sulphate) dissolved in their drinking water during 7 days. In this manner, acute colitis was induced (Kojouharoff *et al.*, 1997). For therapeutic purposes these mice were orally inoculated by means of a gastric catheter using 100µl bacterial suspension (minimum 1.10^8 cells) from day 1 until day 7 of the DSS treatment. Six mice were inoculated with MG1363 [pTREX1] cells (group 1), six mice were inoculated with MG1363 [pT1mTFF1] cells (group 2)

and three mice were inoculated with MG1363 [pT1rTFF3] cells (group 3) and three mice were not inoculated (group 4). On day 8 after the induction of colitis, the mice were sacrificed and examined immunologically and histologically.

Immunological testing of the sera showed that the treated mice did not show an immune response towards the expressed proteins. Serum was taken from the mice which were bled at day 8. This serum was analysed via Western blotting to check whether it contained antibodies against the proteins present in the medium fractions of the *L. lactis* cells. The medium fractions used were derived from the *L. lactis* strains MG1363 [pTREX1]; MG1363 [pT1mTFF1] and MG1363 [pT1rTFF3]. An equivalent of 1 ml of concentrated (phenol extraction and ethanol precipitation) medium fractions were analysed by SDS-polyacrylamide (20%) gel electrophoresis. After blotting to nitrocellulose filters, the filters were incubated for 1 hour with the serum solutions of the 4 groups of mice. The serum was diluted 500 times in 20ml nitrocellulose blocking buffer (Blotto: 100ml 10x PBS, 150ml 1M NaCl, 2ml Triton X-100, 25g fat-free milkpowder, water up to a total volume of 1 liter). As a secondary antibody, sheep anti-mouse IgG coupled to horseradish peroxidase (HRP) was used. Using the 500 times diluted serum, no signal was detected.

Histological analysis was performed on colons of the treated mice. The colons were cut in the length direction and divided in three equal portions: the distal (nearest to the anus), middle and proximal parts. These colon parts were analysed histologically after an overnight fixation in 3.7% formaldehyde (in PBS), followed by paraffin embedding, ensuring upright positioning of the tissue samples in the paraffin blocks. Of each tissue sample, three parallel 3µm thick longitudinal sections, evenly spaced over the sample, were made. These crossections were coloured with hematoxylin/eosin. Histological analysis was performed in a blind fashion, meaning that the labels on the slides were covered before scoring the sections. Slides carrying sections obtained from the several groups of mice were randomized before microscopic examination. Each slide was then assigned a histological score (ranging from 0 to 5) according to the symptomatic description as defined in Table 1.

For each mouse and for each colon part, the average score of the three sections was calculated. In the distal and middle parts of the colon, the

inflammation consisting of epithelial damage and infiltration were the most pronounced. In the proximal part, almost no inflammation could be observed. The average histological score was calculated for both the distal and the middle colon part per group of animals. The final histological sum score is the sum of the two separate scores (sum score = score of epithelial damage + score of infiltration) and is a measure for the degree of the inflammation. The histological sum scores of the middle colon part and the distal colon part for each of the groups of mice is shown in Figures 3 and 4, respectively.

From the histological scores as set out in Figure 3, it could be concluded that the decrease in inflammation of the middle part of the colon is exclusively due to the presence of the *Lactococcus* cells, independent from the production of trefoil peptides. The addition of *L. lactis* cells caused an inflammation decrease of about 40%. This could imply that *L. lactis* cells have an effect on the intestinal microflora which is also at the basis of the inflammation.

From the histological scores for the distal part of the colon as set out in Figure 4, it could be concluded that there is a clear decrease of inflammation upon inoculation of mice with *L. lactis* cells producing trefoil peptides. Mice having received [pT1mTFF1] transformed *L. lactis* cells show a significant reduction of the inflammation of more than 65%. Treatment with pT1rTFF3] *L. lactis* transformed cells caused a reduction of inflammation of about 50%.

As can be seen from Figure 4, the inflammatory infiltration and the epithelial damage in the distal part of the colon are significantly decreased following inoculation with recombinant *L. lactis* strains which secrete either mTFF1 polypeptide or rTFF3 polypeptide.

Score	Epithelium damage	Inflammatory infiltration
0	Normal morphology	No infiltration
1	Loss of a few goblet cells	Infiltration around the basis of the crypts
2	Widespread loss of goblet cells	Infiltration which reaches the Lamina muscularis mucosae
3	Loss of crypts	Extensive infiltration which reaches the Lamina muscularis mucosae and thickening of the mucosa with prominent oedema
4	Widespread loss of crypts	Infiltration which reaches the Lamina submucosa

Table 1. ***Symptomatic description of histological scores.***
Inflammatory infiltration includes infiltration of the granulocytes, macrophages and lymphocytes.

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CLAIMS

1. A recombinant micro-organism, preferably a bacterial strain, preferably a non-pathogenic strain, preferably a non-invasive strain, preferably a food grade strain, preferably a gram-positive bacterial strain, delivering a trefoil peptide *in vivo*.

2. A micro-organism according to claim 1, wherein said gram-positive bacterial strain is a *Lactococcus* or a *Lactobacillus species*.

3. A micro-organism according to claim 1 or 2 wherein said trefoil peptide is an intestinal trefoil polypeptide (TFF3).

4. A micro-organism according to claim 1 or 2 wherein said trefoil polypeptide is TFF1.

5. Method for the delivery of trefoil peptide to the gastro-intestinal tract comprising the administration of a micro-organism according to claims 1 to 4.

6. Pharmaceutical composition comprising a micro-organism according to any of claims 1 to 4.

7. Method of treatment of all types of gastric and intestinal diseases and disorders comprising the administration of a micro-organism according to any of claims 1 to 4.

8. Use of a micro-organism according to any of claims 1 to 4 for the preparation of a medicament for treatment of acute gastro-intestinal inflammatory diseases, such as acute colitis, including but not limited to acute flare-ups of Crohn's disease and ulcerative colitis.

9. Method for producing a micro-organism according to any of claims 1 to 4 comprising transforming a micro-organism with a recombinant vector carrying

a trefoil polypeptide coding sequence under the control of a suitable promoter and a suitable secretion signal sequence.

10. Recombinant vector comprising a trefoil peptide coding sequence under the control of a suitable promoter sequence and a suitable secretion signal sequence.

11. Recombinant vector according to claim 10, having a nucleotide sequence as presented in any of the figures 1b, 1c, 1d and 1e.

ABSTRACT

The present invention relates to a micro-organism, preferably a bacterial strain, preferably a non-pathogenic strain, preferably a non-invasive strain, preferably a food grade strain, preferably a gram-positive bacterial strain, delivering a trefoil peptide *in vivo*. Preferably said trefoil peptide is an intestinal trefoil polypeptide (TFF3) or TFF1. The present invention further relates to a method for the delivery of trefoil peptide to the gastro-intestinal tract comprising the administration of such a bacterial strain. The present invention also relates to a pharmaceutical composition comprising a trefoil peptide delivering bacterium as well as methods of treatment of acute gastro-intestinal inflammatory diseases comprising administration of said transformed bacterial strains, particularly for treating acute colitis, including but not limited to acute flare-ups of Crohn's disease and ulcerative colitis in humans, as well as for treating gastro-intestinal disorders of a similar nature in other animal species.

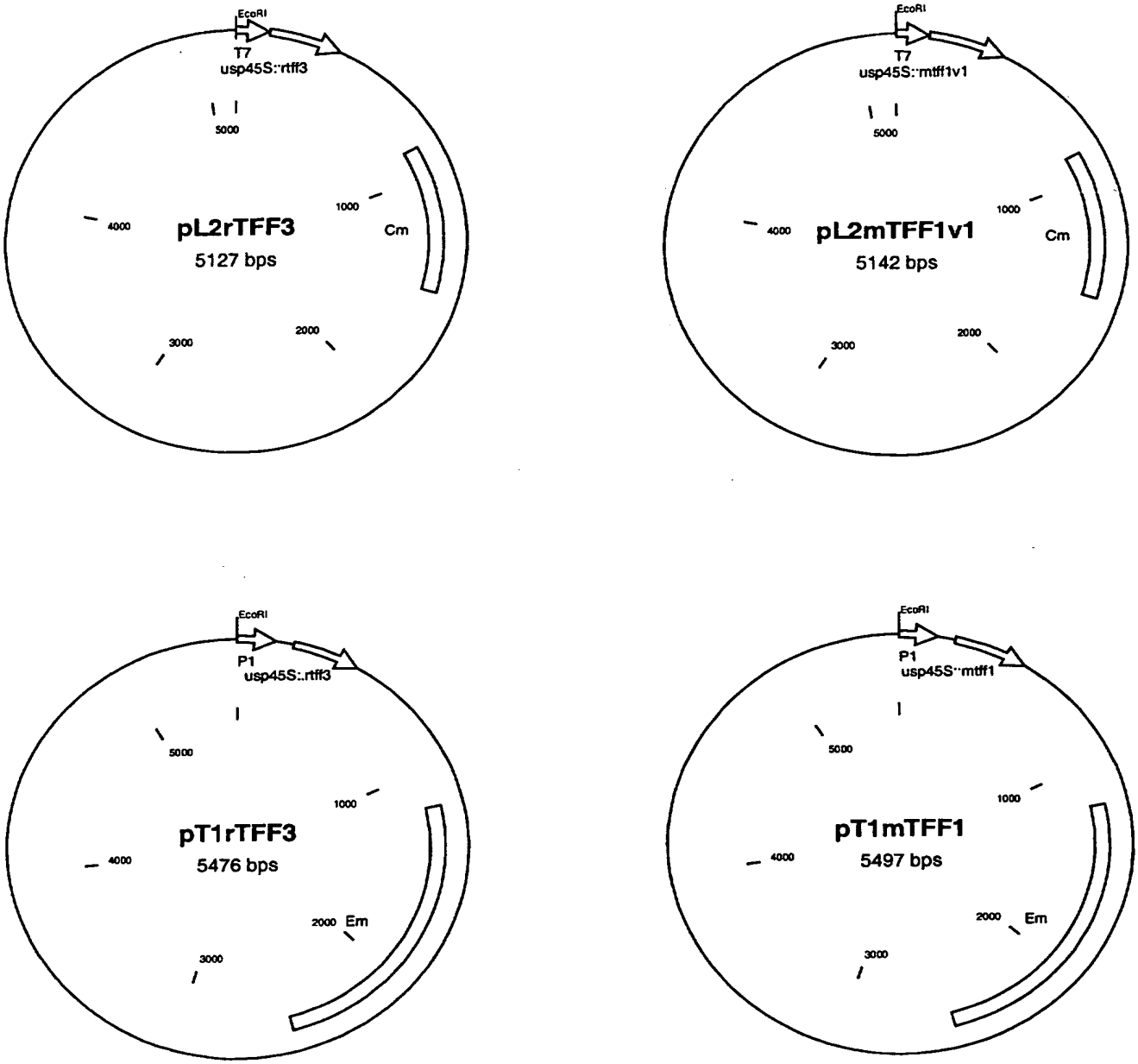
EcoRI
T7
usp45S::rtff3
5000
1000
4000
pL2rTFF3
5127 bps
Cm
3000
2000
EcoRI
T7
usp45S::mtff1v1
5000
1000
4000
pL2mTFF1v1
5142 bps
Cm
3000
2000
EcoRI
P1
usp45S::rtff3
5000
1000
4000
pT1rTFF3
5476 bps
2000
Em
3000
EcoRI
P1
usp45S::mtff1
5000
1000
pT1mTFF1
4000
5497 bps
2000
Em
3000

Figure 1a

Figure 1b :
pL2rTFF3

GAATTCGAGCTCGGTACCCGGGGATCTCGATCCCGCGAAATTAATACGACTC
ACTATAGGGAGACCACAACGGTTTCCCTCTAGAAATAATTTTGTTTAACTTT
AAGAAGGAGATATACATATGAAAAAAAAGATTATCTCAGCTATTTTAATGTC
TACAGTCATACTTTCTGCTGCAGCCCCGTTGTCAGGTGTTTACGCCCAGGAA
TTTGTTGGCCTATCTCCAAGCCAATGTATGGCTCCAACAAATGTCAGGGTGG
ACTGTAACTACCCCACTGTCACATCAGAGCAGTGTAACAACCGTGGTTGCTG
TTTTGACTCCAGCATCCCAAATGTGCCCTGGTGCTTCAAACCTCTGCAAGAG
ACAGAATGTACATTTTAACTAGTAGATCCGGCTGCTAACAAAGCCCGAAAGG
AAGCTGAGTTGGCTGCTGCCACCGCTGAGCAATAACTAGCATAACCCCTTGG
GGCCTCTAAACGGGTCTTGAGGGGTTTTTTGCTGAAAGGAGGAACTATATCC
GGATGACCTGCAGGCATGCAAGCTTGGCACTGGCCGTCGTTTTACAACGTCG
TGACTGGGAAAACCCTGGCGTTACCCAACTTAATCGCCTTGCAGCACATCCC
CCTTTCGCCAGCTGATTTCACTTTTTGCATTCTACAAACTGCATAACTCATA
TGTAAATCGCTCCTTTTTAGGTGGCACAAATGTGAGGCATTTTCGCTCTTTC
CGGCGAGGCTAGTTACCCTTAAGTTATTGGTATGACTGGTTTTAAGCGCAAA
AAAAGTTGCTTTTTCGTACCTATTAATGTATCGTTTTAGAAAACCGACTGTA
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CTGACAATTCCTGAATAGAGTTCATAAACAATCCTGCATGATAACCATCACA
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TCTTGATAATAAGGGTAACTATTGCCGGGATAGACTGTAACATTCTCACGCA
TAAAATCCCCTTTCATTTTCTAATGTAAATCTATTACCTTATTATTAATTCA
ATTCGCTCATAATTAATCCTTTTTTCTTATTACGCAAAATGGCCCGATTTAAG
CACACCCTTTATTCCGTTAATGCGCCATGACAGCCATGATAATTACTAATAC
TAGGAGAAGTTAATAAATACGTAACCAACATGATTAACAATTATTAGAGGTC
ATCGTTCAAAATGGTATGCGTTTTGACACATCCACTATATATCCGTGTCGTT
CTGTCCACTCCTGAATCCCATTCCAGAAATTCTCTAGCGATTCCAGAAGTTT
CTCAGAGTCGGAAAGTTGACCAGACATTACGAACTGGCACAGATGGTCATAA
CCTGAAGGAAGATCTGATTGCTTAACTGCTTCAGTTAAGACCGAAGCGCTCG
TCGTATAACAGATGCGATGATGCAGACCAATCAACATGGCACCTGCCATTGC
TACCTGTACAGTCAAGGATGGTAGAAATGTTGTCGGTCCTTGCACACGAATA
TTACGCCATTTGCCTGCATATTCAAACAGCTCTTCTACGATAAGGGCACAAA
TCGCATCGTGGAACGTTTGGGCTTCTACCGATTTAGCAGTTTGATACACTTT
CTCTAAGTATCCACCTGAATCATAAATCGGCAAAATAGAGAAAAATTGACCA
TGTGTAAGCGGCCAATCTGATTCCACCTGAGATGCATAATCTAGTAGAATCT

Figure 1b – continued -

CTTCGCTATCAAAATTCACTTCCACCTTCCACTCACCGGTTGTCCATTCATG
GCTGAACTCTGCTTCCTCTGTTGACATGACACACATCATCTCAATATCCGAA
TAGGGCCCATCAGTCTGACGACCAAGAGAGCCATAAACACCAATAGCCTTAA
CATCATCCCCATATTTATCCAATATTCGTTCCTTAATTTCATGAACAATCTT
CATTCTTTCTTCTCTAGTCATTATTATTGGTCCATTCACTATTCTCATTCCC
TTTTCAGATAATTTTAGATTTGCTTTTCTAAATAAGAATATTTGGAGAGCAC
CGTTCTTATTCAGCTATTAATAACTCGTCTTCCTAAGCATCCTTCAATCCTT
TTAATAACAATTATAGCATCTAATCTTCAACAAACTGGCCCGTTTGTTGAAC
TACTCTTTAATAAAATAATTTTTCCGTTCCCAATTCCACATTGCAATAATAG
AAAATCCATCTTCATCGGCTTTTTCGTCATCATCTGTATGAATCAAATCGCC
TTCTTCTGTGTCATCAAGGTTTAATTTTTTATGTATTTCTTTTAACAAACCA
CCATAGGAGATTAACCTTTTACGGTGTAAACCTTCCTCCAAATCAGACAAAC
GTTTCAAATTCTTTTCTTCATCATCGGTCATAAAATCCGTATCCTTTACAGG
ATATTTTGCAGTTTCGTCAATTGCCGATTGTATATCCGATTTATATTTATTT
TTCGGTATTTTTTATTAAAACGTCTCAAAATCGTTTCTGGGACGTTTTAGCG
TTTATTTCGTTTAGTTATCGGCATAATCGTTAAAACAGGCGTTATCGTAGCG
TAAAAGCCCTTGAGCGTAGCGTGCTTTGCAGCGAAGATGTTGTCTGTTAGAT
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TTTTTCAAAATCTGCGGTTGCGGCGTTAGCTATAGAAGAATATGCAAGAAAG
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TTTTCGCTACTTGTTTTTGATAAGGTAATATATCATGGCTATTAAAAATACT
AAAGCTAGAAATTTTGGATTTTTATTATATCCTGACTCAATTCCTAATGATT
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AGCCCCCCTGACGAAAGTCGCGACTTCGTTCTTTTTTTACCTCTCGGTTATG
AGTTAGTTCAAATTCGTTCTTTTTAGGTTCTAAATCGTGTTTTTCTTGGAAT
TGTGCTGTTTTATCCTTTACCTTGTCTACAAACCCCTTAAAAACGTTTTTAA
AGGCTTTTAAGCCGTCTGTACGTTCCTTAAG

Figure 1c:
pL2mTFF1v1

GAATTCGAGCTCGGTACCCGGGGATCTCGATCCCGCGAAATTAATACGACTC
ACTATAGGGAGACCACAACGGTTTCCCTCTAGAAATAATTTTGTTTAACTTT
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TTTTGATGACAGTGTCCGGGGATTCCCGTGGTGCTTCCACCCCATGGCCATC
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CTTTGAAGTCATTCTTTACAGGAGTCCAAATACCAGAGAATGTTTTAGATAC
ACCATCAAAAATTGTATAAAGTGGCTCTAACTTATCCCAATAACCTAACTCT
CCGTCGCTATTGTAACCAGTTCTAAAAGCTGTATTTGAGTTTATCAGCCTTG
TCACTAAGAAAATAAATGCAGGGTAAAATTTATATCCTTCTTGTTTTATGTT
TCGGTATAAAACACTAATATCAATTTCTGTGGTTATACTAAAAGTCGTTTGT
TGGTTCAAATAATGATTAAATATCTCTTTTCTCTTCCAATTGTCTAAATCAA
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GATAATTACTAATACTAGGAGAAGTTAATAAATACGTAACCAACATGATTAA
CAATTATTAGAGGTCATCGTTCAAAATGGTATGCGTTTTGACACATCCACTA
TATATCCGTGTCGTTCTGTCCACTCCTGAATCCCATTCCAGAAATTCTCTAG
CGATTCCAGAAGTTTCTCAGAGTCGGAAAGTTGACCAGACATTACGAACTGG
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CGATAAGGGCACAAATCGCATCGTGGAACGTTTGGGCTTCTACCGATTTAGC
AGTTTGATACACTTTCTCTAAGTATCCACCTGAATCATAAATCGGCAAAATA
GAGAAAAATTGACCATGTGTAAGCGGCCAATCTGATTCCACCTGAGATGCAT
AATCTAGTAGAATCTCTTCGCTATCAAAATTCACTTCCACCTTCCACTCACC

Figure 1c – continued –

GGTTGTCCATTCATGGCTGAACTCTGCTTCCTCTGTTGACATGACACACATC
ATCTCAATATCCGAATAGGGCCCATCAGTCTGACGACCAAGAGAGCCATAAA
CACCAATAGCCTTAACATCATCCCCATATTTATCCAATATTCGTTCCTTAAT
TTCATGAACAATCTTCATTCTTTCTTCTCTAGTCATTATTATTGGTCCATTC
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Figure 1d:
pT1rTFF3

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Figure 1d – continued –

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Figure 1d – continued –

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Figure 1e:

pT1mTFF1

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Figure 1e – continued -

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Figure 1e – continued -

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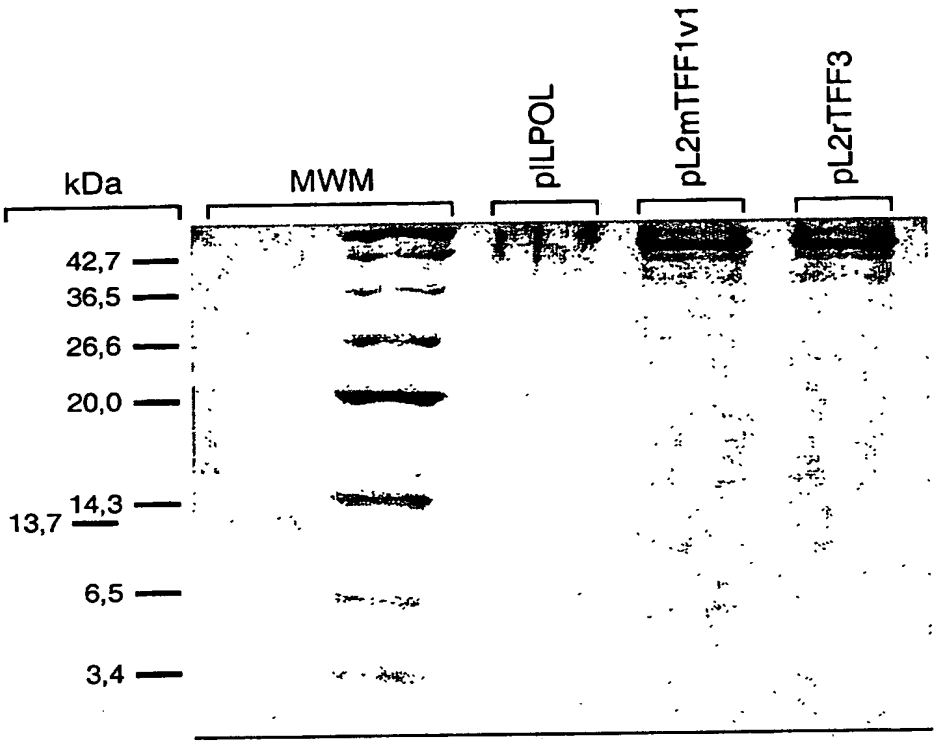
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pILPOL
pL2mTFF1v1
pL2rTFF3
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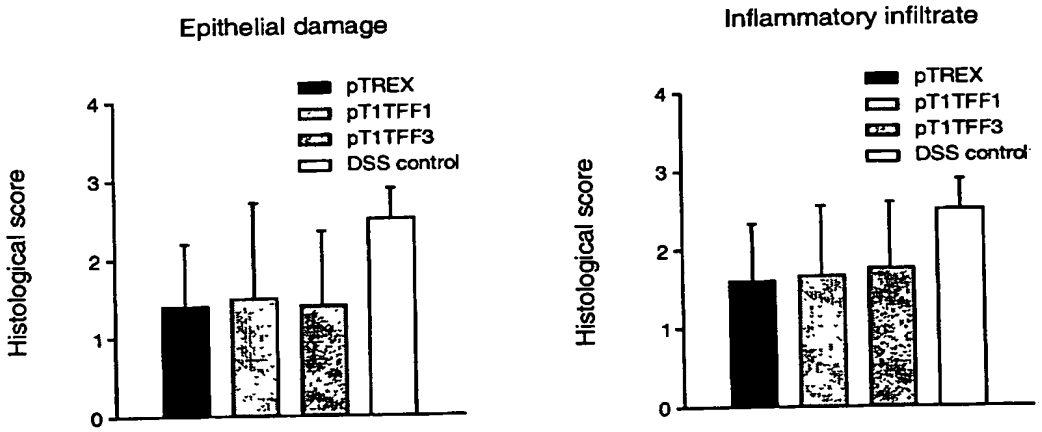
20,0
14,3
13,7
6,5
3,4

Figure 2

Middle Colon



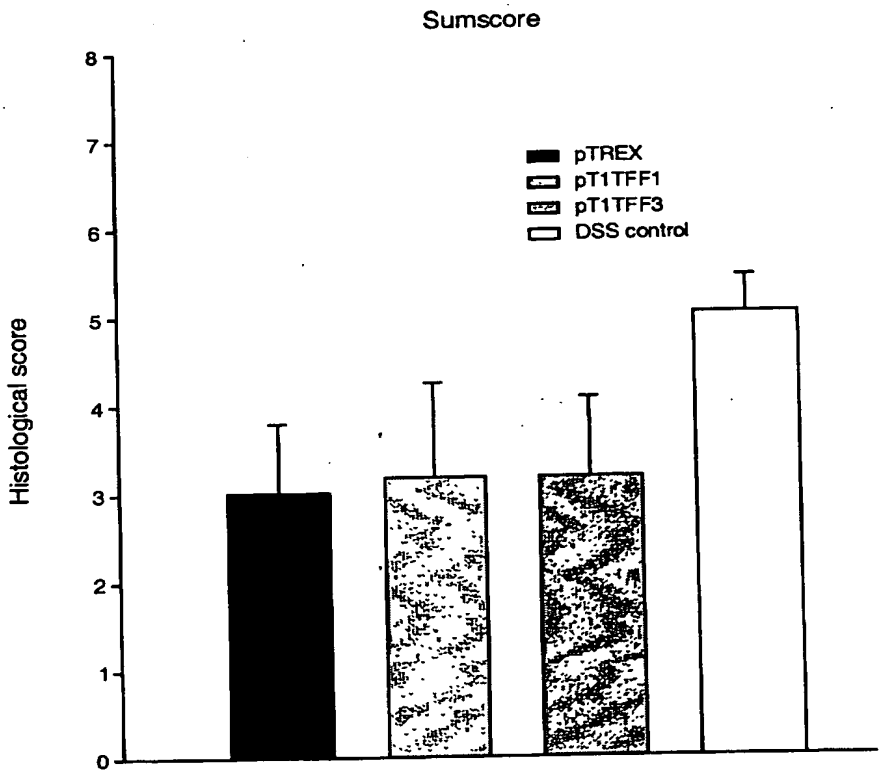
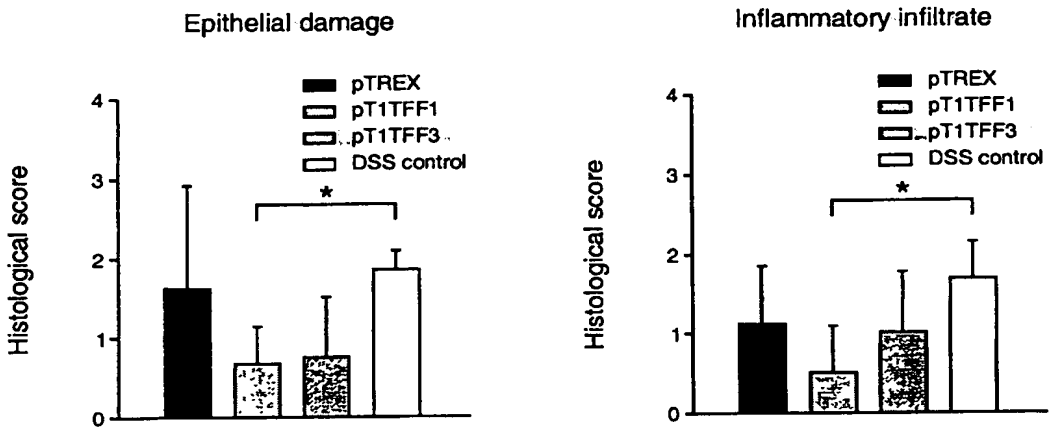


Figure 3

Distal Colon



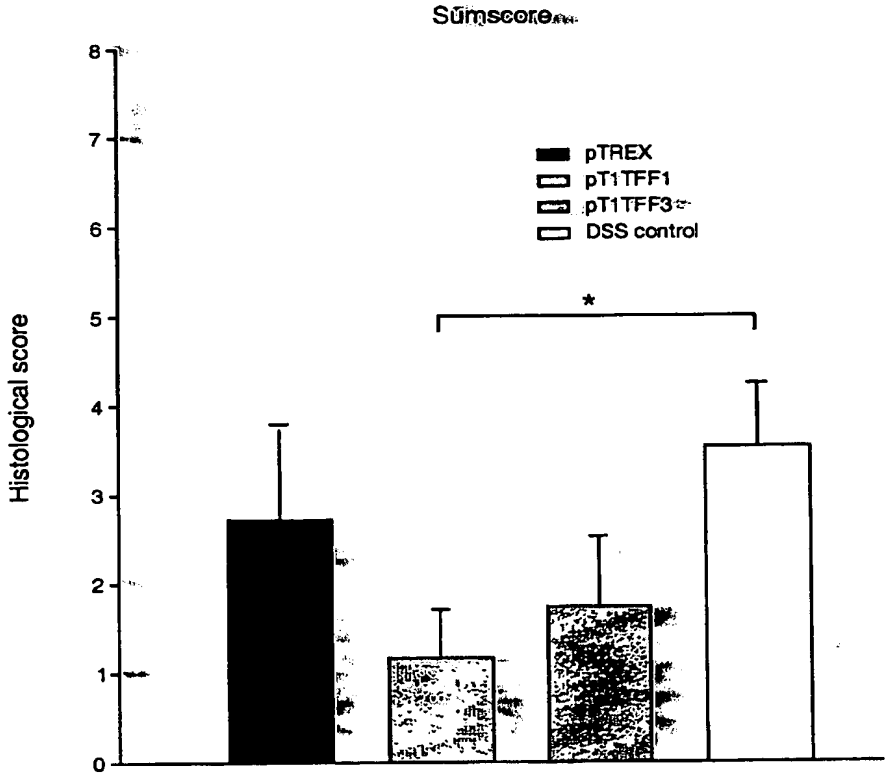


Figure 4